ARMY RESEARCH LABORATORY

Detection of Non-hazardous, Fluorescent Ricin-B Via an Immunoassay on Simulated Plastic Wings

by Glenn Beatty and Asha Hall

ARL-TN-0499 **September 2012**

NOTICES

Disclaimers

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Citation of manufacturer's or trade names does not constitute an official endorsement or approval of the use thereof.

Destroy this report when it is no longer needed. Do not return it to the originator.

Army Research Laboratory
Aberdeen Proving Ground, MD 21005

ARL-TN-0499 **September 2012**

Detection of Non-hazardous, Fluorescent Ricin-B Via an Immunoassay on Simulated Plastic Wings

Glenn Beatty and Asha Hall
Vehicle Technology Directorate, ARL

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED (From - To)
September 2012	Final	July 2012

4. TITLE AND SUBTITLE
Detection of Non-hazardous, Fluorescent Ricin-B Via an Immunoassay on Simulated Plastic Wings

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Glenn Beatty and Asha Hall

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
U.S. Army Research Laboratory
ATTN: RDRL-VTM
Aberdeen Proving Ground, MD 21005

8. PERFORMING ORGANIZATION REPORT NUMBER
ARL-TN-0499

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
As a schedule 1 controlled substance, ricin is one of the deadliest plant toxins in existence, and also one of the most easily weaponizable. This combination has resulted in a recent surge of studies proposing new and improved methods of detecting ricin, primarily involving aqueous phase immunoassays. Successive methods report higher binding affinities and correspondingly lower detection limits, but little research has addressed the potential for new modes of implementation, extending beyond the aqueous phase. This study has examined the potential for detection along a solid/aerosol interface, increasing the variety of prospective scenarios to which the method can be applied. A solid plastic substrate treated with PolySorp™, simulating a plastic wing surface, was used to anchor the potential antibody, a glycosphingolipid called monosialoganglioside (GM1). A monolayer of GM1 was introduced via direct adsorption followed by several rinsing steps, and verification of successful deposition is validated by Raman spectroscopy. The substrate-antibody complex is exposed to a known amount of fluorescently tagged, aerosolized ricin B (non-toxic analog), in a sealed, circulating environment.

15. SUBJECT TERMS
Ricin B, chemical biological detection, immunoassay

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Asha Hall
Unclassified	Unclassified	Unclassified	UU	18	19b. TELEPHONE NUMBER (*Include area code*) (410) 278-8036

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18

Contents

List of Figures

Acknowledgments

This research was supported by an appointment to the Student Research Participation Program at the U.S. Army Research Laboratory (ARL) administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and ARL.

INTENTIONALLY LEFT BLANK.

1. Introduction

As a result of ever-increasing medical knowledge, bioterrorism poses an elevated threat to our modern global community. Consequently, developing onsite field detection devices allowing first responders to detect and mitigate threats is a high priority. Based on criteria developed for the Centers for Disease Control and Prevention, there are three biotoxins posing major threats: botulinum toxin (BTX), ricin, and staphylococcal enterotoxin B (SEB) (*1*).

As a holotoxin, ricin is a protein consisting of multiple subunits. Specifically a dimer, ricin contains two chains, ribotoxin (ricin A chain) and a glactose-binding lectin (ricin B chain). As a type II ribosome inactivating protein (RIP), ricin is a potent toxin, able to inhibit protein synthesis and quickly shut down cellular activity. The presence of ricin B magnifies the toxicity of the protein, binding to glycoproteins on the cell membrane and providing access to the cytosol (liquid component of the cytoplasm surrounding the organelles) (*2*). Ricin A then binds to ribosomes and removes amino acid 28S, irreversibly deactivating ribosomes at a rate upwards of 1500 ribosomes/min (*3*–*5*). This process propagates very quickly and will often progress to serious organ damage, because there is no antidote. The median lethal dosage for inhalation (LD_{50}) is only 22 µg/kg. The toxin is particularly dangerous if inhaled; exposure to a lethal dosage, approximately 2 mg for a 200 lb adult, will typically result in respiratory failure followed by death.

However, it is not the extreme toxicity of ricin, but rather the ease of which the native plant *Ricinus communis* can be obtained that makes ricin an attractive biological weapon. Large quantities of the toxin could potentially wind up in the wrong hands and be armed with relative ease. This danger has spawned many recent studies, reporting new and improved methods of detection, resulting in lower detection limits and novel extraction mechanisms (*5*). The majority of these methods involve immunoassays or enzyme linked immunosorbent assays (ELISA) in aqueous media, so there is some limitation regarding the onsite applicability of these techniques. The goal of this study is to increase the potential modes of implementation for these methodologies by adapting them to operate along a solid/aerosol interface.

The antibody chosen for this study, a glycosphingolipid called monosialoganglioside (GM1), has been found to bind to ricin very effectively (*6*). The two terminal beta galactoside residues on the molecule can each bind with one of two epitopes on ricin B, making it a very effective ligand for capture (*7*). The long, non-polar carbon chains comprising the tail of the molecule are very ideal for adsorbing to many plastics, particularly PolySorp™, which was chosen as the simulated wing surface for this study. The tail of the antibody should hypothetically adsorb to the surface of plastic, exposing the polar carbohydrate moiety to the surrounding atmosphere. This should allow for targeted extraction of any ricin B exposed to the surface.

The objective of this research is to offer a chemical and/or biological (CB) hazard recognition solution in the form of a "receptor skin" for a robotic vehicle. This approach provides a useful tactical advantage for the Army warfighting challenges and Warfighter outcomes (WFO) by addressing force protection and battlespace/situational awareness challenges. The underlying principle is to have a target molecule (i.e., ricin) absorb and diffuse through the skin layer that is on the outside of an unmanned air vehicle (UAV), as shown in figure 1.

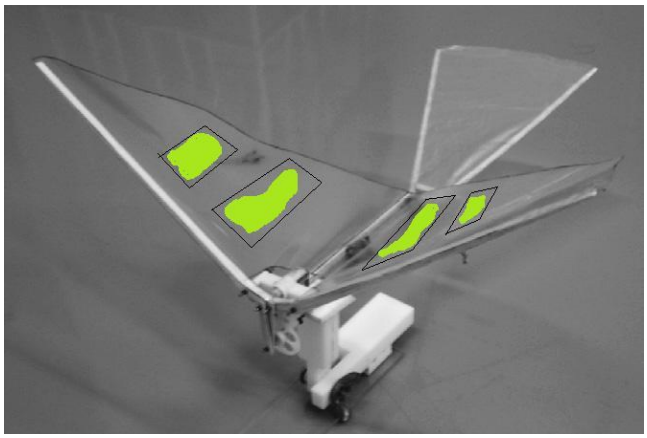

Figure 1. Commercial-off-the shelf flapping wing micro air vehicle with fluorescence tag of ricin B on the surface of the wings.

As a proof of concept, a fluorescent tag was applied to track the non-hazardous ricin B chain (both chains need to be present in order to be classified as a toxin), which poses as the antigen (target) molecule. A relatively dilute solution of the tagged antigen complex was aerosolized and blown through the structure. The fluorescent tag serves to track the mobility of the molecule through the channels of the skin layer and also validates the antibody-antigen binding sequence. A test station was developed to further evaluate hazard detection capability in an experimental setup. Future implementation can be envisioned to integrate the robotic receptor skin onto a variety of pre-existing micro air vehicles (MAVs), UAVs, or unmanned ground vehicles (UGVs).

2. Experimental Procedure

2.1 Reconstitution and Dilution of the GM1 Antibody

This section describes the steps employed to safely, successfully reconstitute, and dilute 1 mg of the GM1 antibody. First, 50 µL of deionized water was added to reconstitute the lyophilized products, mixed gently by inverting 5–6 times at room temperature, and allowed to sit for several minutes (the entirely of the experiment was performed under a fume hood). The reconstituted antibody, GM1, was stored for no more than 2 weeks at 4 °C, but could be potentially stored for

2–3 weeks at 2–8 °C or for up to 12 months at –20 °C or below. Phosphate-buffered saline (PBS) was prepared by diluting 50 mL of Cellgro 10X PBS solution to a final volume of 500 mL; the pH remained constant at ~7.4. Next, 975 µL of PBS buffer was added to GM1, diluting it to a final concentration of 0.5 mg/mL.

2.2 Deposition of GM1 onto PolySorp™ Surface

This section describes the steps employed to safely and successfully deposit the GM1 antibody onto a PolySorp™ substrate. The PolySorp™ array was inspected for any damage or imperfections that could hinder operation. The PolySorp™ surface was cleaned by transferring an appropriate aliquot into each well, using approximately 250 µL of 0.1% Triton-X 100 detergent in PBS buffer for a total of three washes. A drying stage was implemented by using a stream of inert nitrogen gas. Next, 25 µL of the prepared GM1 solution was deposited into each desired well, followed by 225 µL of PBS. After overnight incubation in a refrigerator, the remaining GM1 solution was decanted/aspirated.

2.3 Deposition of Monolayer of Excess GM1

This section describes the steps required to safely and successfully rinse the deposited GM1 to obtain the desired monolayer. Each well was rinsed with a 250-µL aliquot of 0.05% Triton-X 100 detergent in PBS buffer and repeated twice for a total of three rinses. The resultant monolayer was dried with a stream of inert nitrogen gas. Next, sodium azide (0.02 mol%) was added as a preservative While sodium azide is quite toxic, the small amount used by this procedure, coupled with its dilute concentration, keeps the observed concentration below the recommended exposure allowed in the workplace.

3. Results and Discussion

Figures 2 displays the Raman spectra taken of silicon dioxide (SiO_2) compared against a spectrum taken of a concentrated drop of GM1 that was allowed to dry on a SiO_2 wafer. Figure 3 depicts the Raman spectra of GM1; it was characterized to set a baseline for the GM1 measurements. The spectra clearly show distinct peaks for GM1 at wave numbers of 1708.4, 1844.9, 1994.1, and 2238.7, in particular, which were considered indicative of the presence of GM1 in subsequent samples. PolySorp™ samples subjected to a variation of the above deposition method were analyzed with Raman spectroscopy to verify the presence of the antibodies on the substrate. Initially, the spectra matched those of standard PolySorp™ nearly perfectly, i.e., there were no peaks distinguishable from the background spectrum. This instilled doubt as to the effectiveness of the method, and so the concentrations of GM1 implemented were revised upward to the values reported in section 2. In order to proceed with the research, verification that a layer of antibodies was being successfully and reliably deposited on a plastic surface was needed.

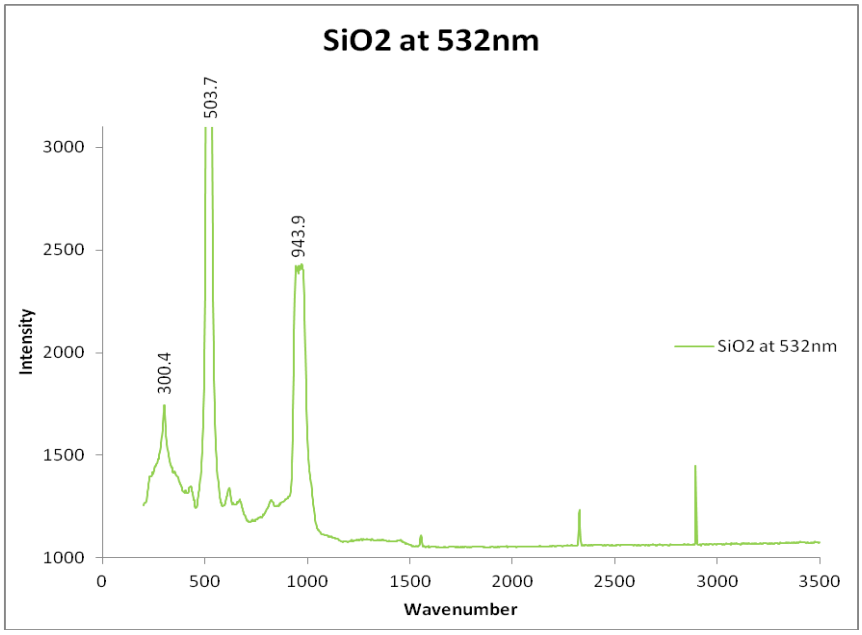

Figure 2. Raman spectrum the same SiO_2 wafer at 532 nm.

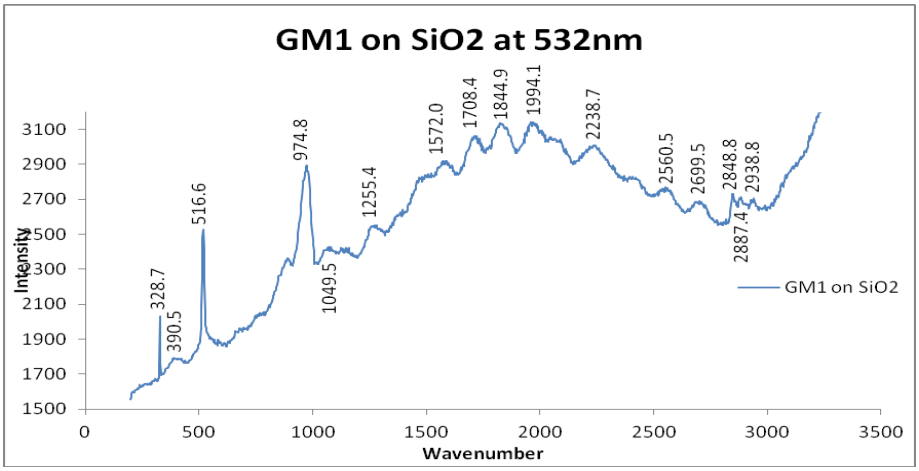

Figure 3. Raman spectrum at 532 nm of concentrated GM1 on a SiO_2 wafer.

After carrying out the revised deposition technique, using solutions at 500 times the previous concentration, the coated plastic was again analyzed using Raman spectroscopy. As depicted in figure 4, the peaks indicative of the background polystyrene were present (as expected) but additional peaks were observed, correlating fairly well with the spectrum observed previously for GM1 on SiO_2. This serves as a positive indication that the deposition technique was successful and that GM1 had effectively adsorbed onto the plastic surface.

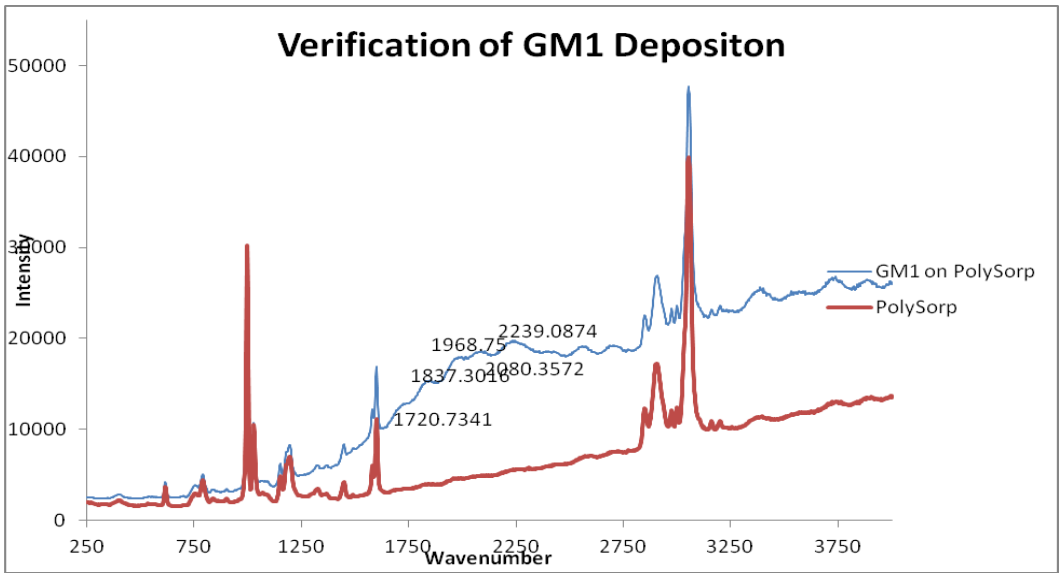

Figure 4. Raman spectra of PolySorp™ compared against PolySorp™ layered with GM1 at 532 nm.

Additionally, the construction of the testing apparatus has been completed, as shown in figure 5. The chamber is sealed and set up with a fan/duct system to re-circulate air, resulting in increased exposure of introduced samples to airborne agents. The aerosol dispersal system (a micro atomizer setup) antibodies have been verifiably coated on the simulated wing surfaces, so their performance can be rapidly assayed.

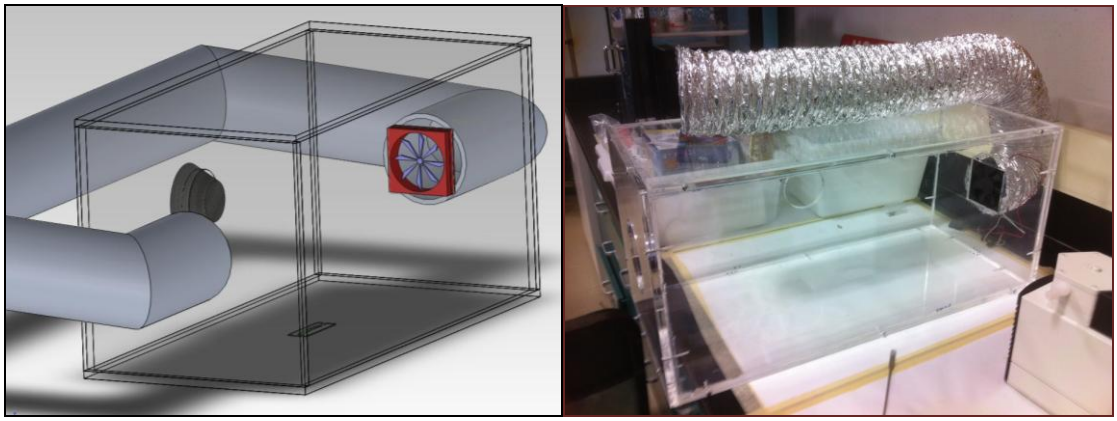

Figure 5. Test chamber schematic (left) and actual setup test chamber (right).

4. Conclusion

This study remains in progress, but the results so far are quite promising. GM1 has been successfully characterized via Raman spectroscopy at 532 nm for the first time, and this spectrum can be used as a reference for future studies involving the compound. GM1 has also

been shown to successfully adsorb to the PolySorp^{TM} surface, which presented the first significant hurdle in the race to selectively extract aerosolized ricin. As a potential continuation, the adherence of GM1 to simple polystyrene will be investigated, to reduce cost and allow for additional modes of characterization such as Fourier transform infrared spectroscopy.

As this research progresses, so will our understanding of how antibodies perform in the presence of aerosols. This is a novel area of research, at least as it pertains to the detection of ricin, and the results are encouraging. Successful extraction of aerosolized ricin B onto a plastic surface, the focus of this study, will bring the U.S. Army one step closer to an autonomous detection method for a high-profile biochemical threat. Such advances aim to achieve the primary objective of the Army: bringing Soldiers home safely.

5. Reference

1. Sano, Y.; Yamashiro, S.; Komano, A.; Maruko, H.; Sekiguchi, H.; Takayama, Y.; Sekioka, R.; Tsuge, K.; Ohsawa, Seto, Y.; Satoh, A. Detection of Proteinous Toxins Using the Bio-Threat Alert System, Part 3: Effects of Heat Pretreatment and Interfering Substances. *Forensic Toxicol* **2007**, *25*, 76–79.
2. Alexander, J. et al. Ricin (From *Ricinus communis*) as Undesirable Substance in Animal Feed. *The EFSA Journal* **2008**, *726*, 1–38.
3. Musshoff, F.; Madea, B. Ricin Poisoning and Forensic Toxicology. *Drug and Testing Analysis* **2009**, *1*, 184–191.
4. Monfort, W. et al. The Three-Dimensional Structure of Ricin at 2.8 A. *Journal of Biological Chemistry* **1987**, *262*, 5398–5403.
5. Day, P. et al. Structure of an Active Site Substitution of Ricin A Chain. *Biochemistry* **1996**, *35*, 11098–11103.
6. Stine, R.; Pishko, M. Comparison of Glycosphingolipids and Antibodies as Receptor Molecules for Ricin Detection. *Analytical Chemistry* **2005**, *77*, 2882–2888.
7. Kortepeter, M.; Parker, G. Potential Biological Weapons Threats. *Emerging Infectious Diseases* **1999**, *5*, 523–527.

List of Symbols, Abbreviations, and Acronyms

BTX	botulinum toxin
CB	chemical and/or biological
ELISA	enzyme linked immunosorbent assays
GM1	glycosphingolipid called monosialoganglioside
MAVs	micro air vehicles
PBS	phosphate buffered saline
RIP	ribosome inactivating protein
SEB	staphylococcal enterotoxin B
SiO_2	silicon dioxide
UAV	unmanned air vehicle
UGVs	unmanned ground vehicles
WFO	Warfighter outcomes

1 (PDF only) DEFENSE TECHNICAL INFORMATION CTR
DTIC OCA
8725 JOHN J KINGMAN RD
STE 0944
FORT BELVOIR VA 22060-6218

1 DIRECTOR
US ARMY RESEARCH LAB
IMAL HRA
2800 POWDER MILL RD
ADELPHI MD 20783-1197

1 DIRECTOR
US ARMY RESEARCH LAB
RDRL CIO LL
2800 POWDER MILL RD
ADELPHI MD 20783-1197

1 DIRECTOR
US ARY RESEARCH LAB
RDRL CIO LT
2800 POWDER MILL RD
ADELPHI MD 20783-1197

ABERDEEN PROVING GROUND

3 US ARMY RESEARCH LAB
ATTN RDRL VTM
D LE
A HALL
M BUNDY
4603 FLARE LOOP
APG MD 21005

1 US ARMY RESEARCH LAB
ATTN RDRL-WMM
J BEATTY
4600 DEER CREEK LOOP
APG MD 21005

INTENTIONALLY LEFT BLANK.